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APPLICATION NUMBER: 60/465,080

FILING DATE: April 24, 2003

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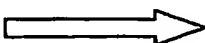
Docket No. 7152

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(e).

04/24/03
J1031 U.S. PTO

JC996 U.S. PTO
60/465080

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TITLE OF THE INVENTION (280 characters max)				
PROMOTERS AND LENTIVIRAL VECTORS FOR EFFICIENT AND COORDINATED EXPRESSION OF MULTIPLE GENES WITHIN EUKARYOTIC CELLS				
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ENCLOSED APPLICATION PARTS (check all that apply)				
<input checked="" type="checkbox"/> Specification Number of Pages <u>16</u>		<input type="checkbox"/> Small Entity Statement		
<input checked="" type="checkbox"/> Drawing(s) Number of Sheets <u>27</u>		<input type="checkbox"/> Other (specify)		
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)				
<input checked="" type="checkbox"/> A check or money order is enclosed to cover the filing fees.		FILING FEE AMOUNT \$160.00		
<input type="checkbox"/> Applicant Claims Small Entity Status				
<input type="checkbox"/> No fee is to be paid at this time.				
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.				
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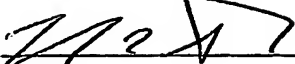
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PROMOTERS AND LENTIVIRAL VECTORS FOR EFFICIENT AND COORDINATED EXPRESSION OF MULTIPLE GENES WITHIN EUKARYOTIC CELLS

The present invention relates to new bidirectional promoters allowing efficient and coordinate expression of two or more genes, to new gene transfer vectors containing these promoters, to the particles transducing said vectors, to the use of said vectors for the delivery and expression of multiple genes in target cells and for the manufacturing of medicaments

INTRODUCTION

Expressing two or more exogenous genes in an efficient and coordinated manner within the same cell is an important but challenging task for gene transfer and gene therapy. The coordinated expression of a marker gene (such as a surface antigen or a reporter gene) together with the gene of interest allows identification of the transfected/transduced cells. By this approach, powerful gene function and target validation studies can be performed in vitro and in vivo, and the ex vivo-transfected/transduced cells can be selected for in vivo administration. By introducing genes encoding drug-resistance or conditional cytotoxicity, the transduced cells can be amplified or eliminated in vivo, respectively. Such strategies would significantly enhance the potential efficacy and/or the safety of gene therapy extending its application to a much larger spectrum of diseases than those currently under study (Kay et al., 2001; Bordignon and Roncarolo, 2002).

In certain gene transfer applications, the coordinated expression of two exogenous genes is essential, for example when the protein to be expressed is made up of subunits encoded by two different genes or when its activity depends on another protein. Typical examples are the followings: co-expression of the two chains of an engineered T-cell receptor or antibody into lympho-hematopoietic cells for adoptive gene transfer; reconstitution of a multi-step enzymatic pathway in deficient or ectopic targets, such as expression of the tyrosine hydroxylase, GTP-cyclohydrolase I and/or DOPA decarboxylase in the striatal neurons of Parkinson's disease; co-expression of synergistic cytokines or antigen plus cytokine in antigen-presenting or other immune

cells for immunotherapy; introduction of multi-component systems for drug-dependent regulation of exogenous gene expression such as the ecdysone, dimerizer and tetracycline-based systems.

To achieve coordinated expression of multiple exogenous genes, several approaches have been tested but the results until now have been less than satisfactory and limited to selected applications. One approach has been to use two separate expression vectors, each of them carrying one of the exogenous genes. However, only a fraction of the target cells are transfected/transduced by the two vectors and a heterogeneous population of cells expressing either one or two genes in different ratio is obtained, often preventing reliable studies and/or efficacious applications. Another approach has been to introduce two expression cassettes driven by different promoters into a single vector (Miller and Rosman, 1989). However, the use of different promoters does not ensure co-expression in the same target cells. Differences in the tissue specificities of the two promoters and interference between their activities often prevent efficient co-expression of the two exogenous genes. Differential splicing may generate different transcripts from the same promoter but it has proven difficult to engineer and adapt to retroviral delivery. A more fruitful approach has been to use an internal ribosome entry site (IRES) sequences (Martinez-Salas, 1999). These sequences, when inserted between two exogenous genes under the control of a single promoter, allow ^{mRNA}Cap-dependent translation of the upstream gene and coupled, Cap-independent translation of the downstream gene in a bicistronic messenger. Here we tested different types of IRES sequences in the context of gene delivery by lentiviral vectors and found significant limitations of this approach. Thus, we developed a new strategy that overcomes these limitations and showed that, upon incorporation into lentiviral vectors, it allows efficient and coordinate delivery and expression of multiple genes.

FIGURE LEGENDS:

Fig.1 Schematic drawings of integrated lentiviral vectors carrying bicistronic expression cassettes. Since transcription from the LTR is abolished due to a deletion in the U3 region, transcription of the internal genes is driven by an internal promoter, here derived from the human cytomegalovirus (hCMV). Bicistronic constructs were

generated by placing an internal ribosome entry site (IRES) between the two genes to be expressed. A wild-type (wt) and mutant (mut) form of the encephalomyocarditis virus (EMCV) IRES, different for the ATG from which downstream translation starts and the eukaryotic IRES derived from the 5' untranslated sequence of the NF- κ B transcription repressing factor (NRF) mRNA were used. The monocistronic vector used for the control experiments is also shown. *Cis*-acting sequences and post-transcriptional regulatory elements enhancing the transduction and expression of the exogenous genes are also indicated. The central polypurine tract (cPPT) upstream of the expression cassettes increases the efficiency of vector transduction. The post-transcriptional regulatory element of the woodchuck hepatitis virus (Wpre) enhances gene expression. For all vectors, the viral packaging signal (Ψ) extended into the beginning of the *gag* gene (GA), the viral splice sites (SD and SA), the Rev response element (RRE) and the U3-deleted LTR are also shown.

Fig.2 Southern analysis of HeLa cells transduced by bicistronic and control monocistronic lentiviral vectors. HeLa cells were infected with matched amounts of lentiviral vector particles carrying the indicated vectors. After serial passages, the HeLa DNA was extracted, digested with Afl-II to release the expression cassette from integrated vector DNA and analysed with a Wpre probe to detect vector sequences. The average number of integrated vector copies was determined relative to the indicated standard curve. This number was used to normalize vector stocks for all subsequent transduction experiments and ensure similar levels of integration for each vector tested.

Fig. 3 Gene expression by bicistronic lentiviral vectors. HeLa and primary human umbilical vein endothelial cells (HUVEC) were transduced with normalized amounts of the indicated vectors expressing luciferase and GFP (2×10^6 TU/ml). After 5 days, expression of both genes was tested by luminescence of cell extracts upon luminol addition (left panel; net luminescence over background, in arbitrary units, A.U.) and by GFP fluorescence of individual cells by FACS (right panel; the percentage of GFP+ cells is indicated with the mean fluorescence intensity, X). Expression of the upstream luciferase gene in bicistronic vectors varied with the IRES type and, only in the case of the EMCV wt was comparable to that achieved by a monocistronic CMV

vector. Expression of the downstream GFP gene also varied with the IRES type and was much less efficient than that observed with the cap-dependent, monocistronic CMV vector.

Fig. 4 Gene expression by bicistronic lentiviral vectors. Human peripheral blood lymphocytes (hPBL), activated by anti-CD3 and anti-CD28 treatment, and cord blood CD34 hematopoietic progenitors were transduced and analysed as in Fig. 3 above, (2×10^7 TU/ml). As previously observed with HeLa and HUVEC, but even more dramatically, IRES-dependent expression was much less efficient than cap-dependent expression from monocistronic vectors. At variance with HeLa, HUVEC and CD34 cells, the only bicistronic vector detectably expressing the IRES-dependent gene in hPBL was the NRF.

Fig.5. Schematic drawings of integrated lentiviral vectors carrying bidirectional expression cassettes. Since transcription from the LTR is abolished due to a deletion in the U3 region, transcription of the internal genes is driven by the internal promoters. The indicated bidirectional promoters were generated by joining a minimal promoter obtained from the cytomegalovirus (minCMV) or mouse mammary tumor virus (minMMTV) and the efficient human PGK promoter (hPGK) in the opposite orientation. They were inserted between two reporter genes and mediate the divergent transcription of two mRNAs, one in the sense and the other one in the antisense orientation relative to the vector (arrows). *Cis*-acting sequences and post-transcriptional regulatory elements enhancing the transduction and expression of the exogenous genes are also indicated. The central polypurine tract (cPPT) upstream of the expression cassettes increases the efficiency of vector transduction. The post-transcriptional regulatory element of the woodchuck hepatitis virus (Wpre) enhances expression of gene 2 and the constitutive transport element of the monkey Mason-Pfizer virus (CTE) enhances expression of gene 1. In addition, the antisense expression cassette includes the polyadenylation site of the simian virus 40 (SV40 polyA).

The monodirectional vectors used for the control experiments are also shown. They comprise vectors carrying a hPGK-driven expression cassette in either orientation and containing the same set of regulatory elements described above, and vectors carrying expression cassettes driven by the two minimal promoters joined to Tet operator

sequences. The latter vectors were used without Tet-dependent activation to assess the basal transcriptional activity of the minimal promoters.

For all vectors, the viral packaging signal (Ψ) extended into the beginning of the *gag* gene (GA), the viral splice sites (SD and SA), the Rev response element (RRE) and the U3-deleted LTR are also shown.

Fig. 6 Gene expression by bidirectional lentiviral vectors. Performance of the first expression cassette, placed in antisense orientation relative to the vector. HeLa cells were transduced with normalized amounts of the indicated vectors expressing luciferase and GFP (10^5 TU/ml). After 5 days, luciferase expression was measured by luminescence of cell extracts upon luminol addition (net luminescence over background, in arbitrary units, A.U.). Gene expression from the PGK promoter was not affected by the joining of the minimal promoter in reverse orientation, as shown by comparing luciferase expression from the monocistronic hPGK vector and the bi-directional MA-2 vector. On the other hand, gene expression from the minimal promoter was significantly enhanced by its joining to the PGK promoter in reverse orientation, as shown by comparing luciferase expression from the uninduced, (TetO) minCMV vector and the bi-directional MA-1 vector.

Fig. 7 Gene expression by bidirectional lentiviral vectors. Performance of the second expression cassette, placed in sense orientation relative to the vector. HeLa cells were transduced with the indicated, normalized amounts of vectors expressing luciferase and GFP. After 5 days, GFP expression was measured by fluorescence of individual cells by FACS (the percentage of GFP+ cells is indicated with the mean fluorescence intensity, X). As observed in Fig.6 for the first cassette, gene expression from the PGK promoter was not affected by the joining of the minimal promoter in reverse orientation, as shown by comparing GFP expression from the monocistronic hPGK vector and the bi-directional MA-1 vector. On the other hand, gene expression from the minimal promoter was significantly enhanced by its joining to the PGK promoter in reverse orientation, as shown by comparing GFP expression from the uninduced, (TetO) minMMTV vector and the bi-directional MA-2 vector.

Fig. 8 Comparison of bidirectional and bicistronic lentiviral vectors. HeLa cells were transduced with the indicated, normalized amounts of bi-directional MA-2 vector and of the best performing IRES wt bicistronic vector, both expressing luciferase and GFP. Expression of both genes was measured as in the preceding figures. As expected from the normalization of transduction, expression of the first gene, luciferase, driven by the PGK promoter in the bidirectional vector and cap-translated in the bicistronic vector, was similar. Strikingly, however, expression of the second gene, GFP, driven by the minimal promoter in the bidirectional vector and IRES-translated in the bicistronic vector, was observed in a much higher fraction of cells with the bidirectional vector.

Fig. 9 Efficient and coordinated gene in human hematopoietic progenitor cells by bidirectional lentiviral vectors. CD34+ hematopoietic progenitors were purified from cord blood and transduced with normalized amounts of the indicated vectors (5×10^7 TU/ml) expressing a truncated form of the human low-affinity NGF receptor (Δ LNGFR) and GFP. The bidirectional MA-1 vector was compared to the best performing IRES wt bicistronic vector and to monocistronic PGK vectors expressing Δ LNGFR or GFP. After 7 days in culture in conditions maintaining the immature progenitors (>80% CD34+), gene expression was measured by Δ LNGFR immunostaining (using phycoerythrin-conjugated antibodies) and FACS analysis. Simultaneous analysis of Δ LNGFR and GFP expression in the top panel shows the fraction of cells expressing only Δ LNGFR (left upper quadrant) or both genes (right upper quadrant), and their mean levels of expression (Y for Δ LNGFR and X for GFP). The MA-1 vector reached a high frequency of Δ LNGFR+ cells with an average expression level similar to that obtained by the monocistronic PGK vector; remarkably, the vast majority of these cells also expressed GFP to high levels. On the other hand, the bicistronic vector reached a lower frequency and a lower level of expression of Δ LNGFR and even more for GFP. The bottom panel shows non-immunostained cells to better visualize the total GFP+ population in comparison with cells transduced with monocistronic PGK vector.

Fig. 10 Maintenance of gene expression upon differentiation of hematopoietic progenitors transduced by bidirectional lentiviral vectors. The same cells described in Fig. 9 were cultured for 14 days in conditions promoting differentiation along the monocytic lineage (>90% CD13+) and then analysed for Δ LNGFR and GFP gene expression. The gene expression patterns described above were maintained upon differentiation.

Fig. 11 Efficient and coordinated gene expression in human lymphocytes cells by bidirectional lentiviral vectors. Human peripheral blood lymphocytes were purified by Ficoll gradient and transduced with normalized amounts (5×10^7 TU/ml) of the indicated vectors either after 4-day treatment with interleukin-7 (Resting) or after 2-day activation with anti-CD3 and anti-CD28 antibodies (Activated). The bidirectional MA-1 vector allowed efficient and coordinated expression of the two marker genes, Δ LNGFR and GFP, analyzed as described in the preceding figures, in both cellular conditions.

Fig 12a Map of the plasmid containing the lentiviral vector construct RRL-MA1-lucif/GFP

Fig. 12b Sequence of the plasmid containing the lentiviral vector construct RRL-MA1-lucif/GFP

Fig. 13a Map of the plasmid containing the lentiviral vector construct CCL-MA1-GFP/deltaLNGFR

Fig. 13b Sequence of the plasmid containing the lentiviral vector construct CCL-MA1-GFP/deltaLNGFR

Fig. 14a Map of the plasmid containing the lentiviral vector construct RRL-MA2-lucif/GFP

Fig. 14b Sequence of the plasmid containing the lentiviral vector construct RRL-MA2-lucif/GFP

Fig. 15a Map of the plasmid containing the lentiviral vector construct CCL-MA3-GFP/deltaLNGFR

Fig. 15b Sequence of the plasmid containing the lentiviral vector construct CCL-MA3-GFP/deltaLNGFR

RESULTS

We first constructed and evaluated bicistronic lentiviral vectors containing different IRES, both of viral and eucaryotic origin, to express two marker genes (Fig. 1). We transduced bicistronic and control, monocistronic vectors into a panel of continuous and primary cells, including human umbilical vascular endothelial cells, hematopoietic progenitors and peripheral blood lymphocytes. To normalize transduction by the different vectors, we transduced HeLa cells with equal amounts of vector particles (as measured by HIV-1 Gag immunocapture) and analysed the transduced cells by Southern blot to calculate the average amount of integrated vectors (fig. 2). We then used this number to normalize the vector stocks and transduce equal amounts of transducing particles into the target cells. By comparing cells transduced by bicistronic and monocistronic vectors, we found that only a fraction of the transduced cells expressed both genes and that the IRES-dependent gene was expressed to significant lower levels than when it was expressed by cap-dependent translation (Fig. 3 and 4). In addition, IRES sequences displayed cell-type specific activity, with different downstream gene expression levels in different cell types. For instance, the IRES derived from the 5' untranslated region of the eucaryotic NF-kB repressive factor mRNA (NRF; Oumard et al., 2000) was the most efficient in lymphocytes (Fig. 4) while one of the IRES derived from the encephalomyocarditis virus (EMCV) was the most efficient in endothelial cells (Fig. 3). In conclusion, these experiments indicated that selection of transduced cells for expression of the IRES-dependent, downstream gene was required to ensure coordinated expression of both genes in all target cells. Although this requirement can be met in some types of applications, it seriously limits the usefulness of bicistronic vectors in most other ones.

To overcome these limitations, we constructed and tested new promoter designs for multiple gene expression and incorporated them into state-of-the-art, self-inactivating lentiviral vectors (Follenzi et al., 2000) to reach stable integration and efficient, coordinated expression in all transduced cells. We joined a minimal promoter upstream to an efficient promoter and in the opposite orientation. We constructed and tested different bi-directional promoters coupling a 400 bp fragment from the human phosphoglycerate kinase promoter with different minimal promoters derived from the cytomegalovirus (CMV) and mouse mammary tumor virus (MMTV) genomes (fig. 5). The upstream expression cassette (in anti-sense orientation relative to the vector) included an exogenous polyadenylation site and a post-transcriptional regulatory element different from the one incorporated in the sense expression cassette to improve expression. By comparing the expression of equal amounts of lentiviral vectors carrying the combined or the separate cassettes, we found that the bi-directional design significantly enhanced transcription from the minimal promoter while not affecting downstream expression from the PGK promoter (fig. 7). Strikingly, this new type of vector enabled efficient and coordinated expression of two genes in the vast majority of transduced cells, outperforming the IRES-based design both when normalized to vector copy number and when assessed for maximal efficiency of expression (fig. 8). We stringently verified these findings using well-quantifiable cell-associated markers such as luciferase, GFP and Δ LNGFR and proving stable co-expression of two genes within the same cell from a single-copy integrated bi-directional vector (fig. 9). We then established the improved performance of the new vectors in primary human hematopoietic progenitors tested as immature progenitors (fig. 10), and after differentiation (fig. 11), and in resting and activated lymphocytes (Fig 12). Lentiviral vectors carrying the bi-directional expression cassettes were produced to high titers and infectivity and transduced efficiently all target cell types tested. We are currently performing murine transplantation studies to verify the potential advantages of the new bi-directional vectors for the amplification and/or selection of a polyclonal population of engineered hematopoietic stem cells. We are also performing vector transgenesis experiments to prove the efficient and coordinated expression of two exogenous genes in all types of tissues.

EXAMPLES

Construction of lentiviral vector with bi-directional promoters

To generate the lentiviral construct **RRL-MA1**, an XhoI-XhoI fragment containing the SV40polyA.CTE.Luciferase.minhCMV elements (derived from the lentiviral construct

pRRL.sin.cPPT.SV40polyA.CTE.Luciferase.minhCMV.TetO7.minMMTV.eGFP)

was cloned into the lentiviral vector construct

pRRL.sin.cPPT.hPGK.eGFP.Wpre(Follenzi et al., 2000) cut with the same enzyme to obtain

RRL-MA1-lucif/GFP

(pRRL.sin.cPPT.SV40polyA.CTE.Luciferase.minhCMV.hPGK.eGFP.Wpre).

To generate the lentiviral construct **CCL-MA1**, two fragments were cloned into the lentiviral construct pRRL.sin.cPPT.hPGK.ALNGFRWpre first cut with KpnI, blunted and then cut with XhoI, the first fragment containing the minhCMV.eGFP elements was derived from the lentiviral construct

pRRL.sin.cPPT.SV40polyA.CTE.Luciferase.minMMTV.TetO7.minhCMV.eGFP cut with KpnI, blunted and then with XhoI and the second derived from the construct pRRL.sin.cPPT.SV40polyA.CTE.tTA2.Wpre cut with BamHI, blunted and then cut with NotI. The resulting lentiviral construct

pRRL.sin.cPPT.SV40polyA.CTE.Luciferase.minMMTV.TetO7.minhCMV.eGFP was cut with NotI and AvrII and the fragment containing the cPPT.SV40polyA.CTE.eGFP.minhCMV.hPGK.ALNGFRWpre was cloned into the lentiviral construct pCCL.sin.cPPT.hPGK.eGFP.Wpre cut with the same enzymes to obtain

CCL-MA1-GFP/ALNGFR

(pCCL.sin.cPPT.SV40polyA.CTE.eGFP.minhCMV.hPGK.ALNGFRWpre).

To generate the lentiviral construct **RRL-MA2**, a HindIII-BamHI fragment containing the hPGK.Luciferase elements (derived from the lentiviral vector construct pRRL.sin.cPPT.hPGK.Luciferase.IRES.Wpre) was cloned into the retroviral construct SF2-cLCM2G (obtained from Rainer Loew, University of Heidelberg, FRG) cut with the same enzymes to obtain the construct

cPPT.SV40polyA.CTE.Luciferase.hPGK.minMMTV.eGFP. This construct was first cut with SalI, blunted and then cut with BamHI and the fragment containing the Luciferase.hPGK.minMMTV.eGFP elements was cloned into the lentiviral vector construct pRRL.sin.cPPT.SV40polyA.CTE.tTA2.Wpre cut in the same way, to obtain RRL-MA2-lucif/GFP (pRRL.sin.cPPT.SV40polyA.CTE.Luciferase.hPGK.minMMTV.eGFP.Wpre).

To generate the lentiviral construct CCL-MA3, two fragments were cloned into the pBLKS+ cut with HindIII and XhoI, the first fragment containing the CTE.SV40polyA elements was derived from the lentiviral vector construct pRRL.sin.cPPT.SV40polyA.CTE.tTA2 cut with HindIII and XbaI and the second fragment containing the minMMTV.GFP elements derived from the construct cPPT.SV40polyA.CTE.Luciferase.hPGK.minMMTV.eGFP cut with XhoI and XbaI to obtain the construct pBLKS+ minMMTV.GFP.CTE.SV40polyA. The resulting construct was cut with EcoRV and XhoI and the fragment containing the minMMTV.GFP.CTE.SV40polyA was cloned into the lentiviral vector construct pCCL.sin.cPPT.hPGK.ΔNGFR.Wpre cut with the same enzymes, to obtain the final lentiviral vector construct CCL-MA3-GFP/ΔNGFR (pCCL.sin.cPPT.SV40polyA.CTE.GFP.minMMTV.hPGK.ΔNGFR.Wpre).

The maps and the nucleotide sequences of the RRL-MA1-lucif/GFP, CCL-MA1-GFP/ΔNGFR, RRL-MA2-lucif/GFP; CCL-MA3-GFP/ΔNGFR constructs are shown respectively in figures 12a-15a and figures 12b-15b.

DISCUSSION

Our results show several important limitations of IRES-containing bicistronic vectors for the co-ordinate delivery and expression of two genes within the same cell. First, for all types of IRES tested, the IRES-dependent gene was expressed to much lower levels than the Cap-dependent gene. Second, the IRES sequences showed cell type-specific differences in translation efficiency, suggesting that different cellular factors interact with the IRES. For example, the NRF IRES was the most efficient sequence in lymphocytes but not in the other cell types tested, probably because it is involved in

the regulation of cytokine translation in these cells. Third, the introduction of an IRES sequence had a negative influence on the expression of the upstream gene. The extent of this phenomenon varied with the target cell type and with the IRES sequence and probably reflects the influence of the IRES secondary structure on the mRNA stability or function. Fourth, because the two genes in a bicistronic expression construct share a common transcript, sequence-specific effects on expression extend from one to both genes. For instance, the bicistronic vectors containing luciferase as upstream gene expressed the IRES translated gene at lower levels as compared to those obtained when the Δ LNGFR was the upstream gene. In addition, by this approach, the expression ratio between the two transgene is dictated by the efficiency of the selected IRES sequence and there is little room for adjusting it. For all these reasons, the majority of transduced cells that expressed the upstream gene in our experiments did not co-express the IRES-dependent gene to detectable levels. Thus, to ensure co-expression of both genes, one must either select the cells for expression of the IRES-dependent gene or transduce multiple copies of vectors in each target cell. Both of these approaches are challenging in most *in vitro* and *in vivo* gene transfer applications.

The new bi-directional promoter design that we have developed and the vectors that incorporate them overcome these limitations. We joined a minimal promoter upstream to an efficient promoter and in the opposite orientation. The rationale behind this design is the sharing of orientation-independent enhancer activity contributed from the efficient promoter between the two closely linked basal promoters acting in opposite directions. The bi-directional promoter thus mediates the coordinated, divergent transcription of two mRNAs. Our experiments showed that lentiviral vectors carrying bi-directional promoters enable efficient and coordinated expression of two genes in the vast majority of transduced cells, outperforming IRES-containing vectors both when normalized to vector copy number and when assessed for maximal efficiency of expression. In addition, the percentage of cells expressing both genes after transduction with a bi-directional vector was comparable to the percentage of cells expressing either transgene after transduction with control monocistronic vectors. By ensuring single copy vector integration in target cells, we also proved that divergent transcription occurred from the same promoter to adequate levels for

reaching coordinate expression of both genes within the same cell. Importantly, the bi-directional promoters were consistently expressed in all types of target cells tested and our long-term analysis showed that this design was neither silenced nor randomly fixed in one direction of transcription, even after cellular differentiation, as in the case of monocyte differentiation from human CD34+ hematopoietic progenitors.

Whether the bi-directional promoter configuration allowed stable transcription of two genes from a single transfected/transduced construct, and whether the two genes reached robust levels of expression could not be anticipated. Moreover, we had to make this design compatible with efficient packaging, transfer and expression by lentiviral vectors, possibly a difficult task given the well-known interference of antisense transcription with retroviral vector production and expression. In nature, few instances of bi-directional promoters have been documented. Artificial bi-directional promoters have been previously used to express two genes under the control of the tetracycline-dependent expression system (Baron et al., 1995). In this design, two copies of a minimal promoter were joined in opposite orientation to both sides of a series of Tet-operator repeats. This expression system used a combination of prokaryotic and eukaryotic elements to achieve exogenously regulated gene expression. To our knowledge no application of bi-directional promoters has been described to the constitutive, ubiquitous or tissue-specific expression of more than one exogenous gene in animal cells. However, bidirectionalization of polar promoters for gene expression in plants has been described (Xie et al., 2001). Given the substantial evolutionary distance between plants and animals it was not known whether this type of approach would also work in animal cells and how to select and engineer the appropriate promoter elements. In addition, the substantial difference between DNA transfer techniques and their outcome in plant genetic engineering versus those used for animal cell genetic engineering hampers cross-predicting the suitability of a gene expression method or construct from one to the other application.

In principle any minimal or "core" promoter, here defined as the DNA sequence region immediately upstream to the transcription initiation site of an artificial or naturally occurring viral or cellular promoter which contains binding sites for basal transcription factors and can initiate downstream transcription only to basal or minimally detectable levels of its own, could be joined as described in the examples to

a more extended promoter sequence, again artificial or naturally occurring viral or cellular, that can drive transcription to well-detectable levels in most cell types or, alternatively, only in specific cell types, to obtain a bi-directional promoter. Although we did not map how close the two promoters must be for operational linkage, we may expect that close juxtaposition of the minimal promoter to some of the upstream elements in the efficient promoter, as observed in natural promoter between core elements and upstream elements, may be required. The minimal CMV and MMTV promoters that we tested are quite different between themselves, for instance the former contains a well-identifiable TATA box while the latter one does not, so it is likely that most other types of minimal or core promoters would similarly work in a bi-directional context. We expect the same to be true for the efficient promoter represented by the PGK promoter in our examples. The expected versatility in bi-directional promoter assembly may allow reaching different expression levels and ratio of the two exogenous genes by exchanging their position or by changing either component of the bi-directional promoter. In addition, the incorporation of post-transcriptional regulatory elements in either or both orientations, as shown in our examples, may further enhance gene expression.

We expect that the bi-directional design described here by engineering the ubiquitously expressed PGK promoter be successfully applied to tissue-specific promoters to obtain coordinated expression of two genes in specific types of tissues. In addition, by combining the use of bi-directional promoters and bicistronic transcripts one could express more than two exogenous genes within the same cells although with the limitations of efficiency described above for the IRES-dependent ones.

The successful generation of lentiviral vectors allowing efficient and coordinated expression of two genes provides the best means to address the requirements of experimental gene transfer and safe and efficacious gene therapy mentioned above. In addition, the bidirectional promoters, and the new lentiviral vectors incorporating them, allow generating transgenic lines co-ordinately expressing multiple transgenes. If adapted for the expression of short interfering RNA, they may also enable coordinate knock-down of multiple genes.

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The application intends to protect:

1. A bidirectional promoter for expression of two or more genes essentially comprising a minimal promoter sequence upstream to a full efficient promoter sequence, this full efficient promoter sequence including an enhancer region, being the two promoter sequences in the opposite orientation.
2. The bidirectional promoter according to Claim 1 wherein the full efficient promoter is derived from the human phosphoglycerate kinase promoter.
3. The bidirectional promoter according to Claim 1 or 2 wherein the minimal promoter is derived from cytomegalovirus (CMV) or mouse mammary tumor virus (MMTV) genomes.
4. A bidirectional expression cassette essentially comprising the bidirectional promoter according to any of previous claims, convenient insertion sites positioned downstream to each promoter, and polyadenylation sites positioned downstream to each insertion site.
5. The bi-directional expression cassette according to claim 4 further comprising at least one post-transcriptional regulatory element positioned upstream to one or each polyadenylation site.
6. The bidirectional expression cassette according to claim 4 or 5 further comprising at least one internal ribosome entry site (IRES) sequence to express three or more genes.
7. An expression construct containing the bidirectional promoter according to claims 1-3.
8. An expression construct containing the bidirectional expression cassette according to claims 4-6.
9. A gene transfer expression vector containing the expression construct according to claims 7 or 8 further comprising lentiviral sequences.
10. Use of the gene transfer expression vector according to claim 9 for the delivery and expression of multiple genes in eukaryotic cells.
11. Method for generating a transgenic non human organism comprising the step of transforming appropriate cells by means of the gene transfer expression vector according to claim 9.

Fig.1 Bicistronic Lentiviral Vectors

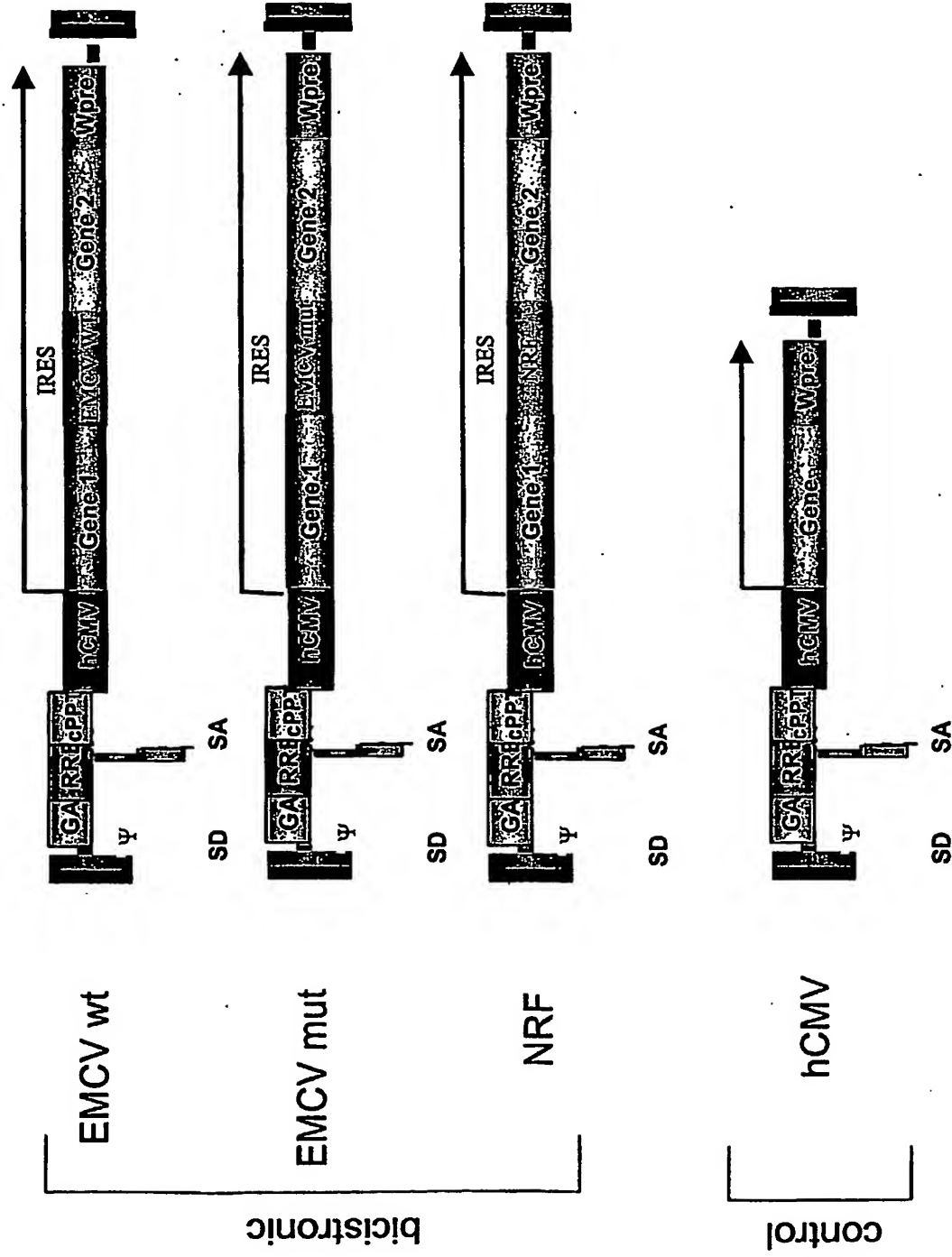
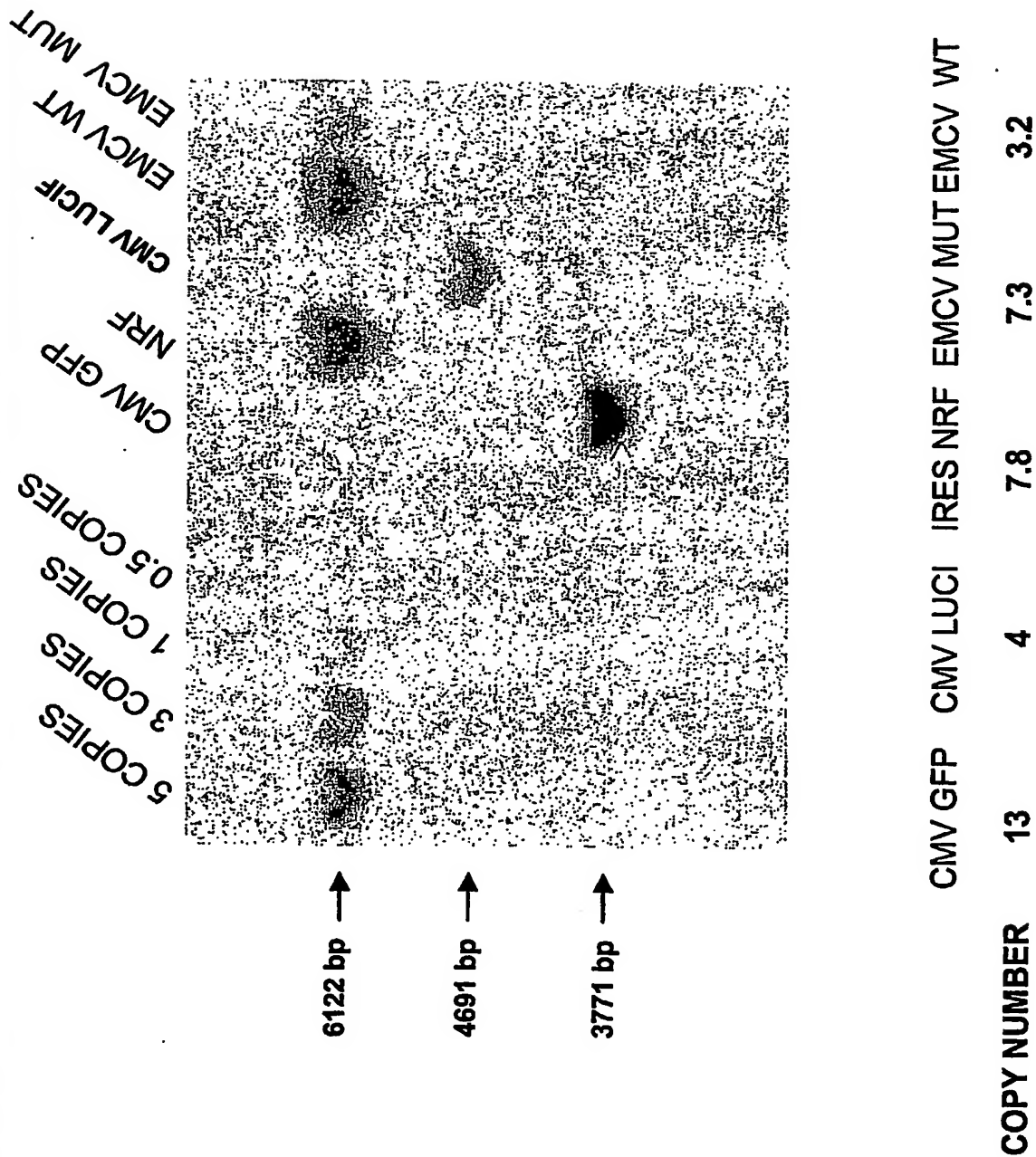


Fig. 2 LV Integration Analysis in Target Cell Genome



ထိုသို့အားဖြင့် နေရာအနှံ့အပြား၌



Fig.5 Bidirectional Lentiviral Vectors

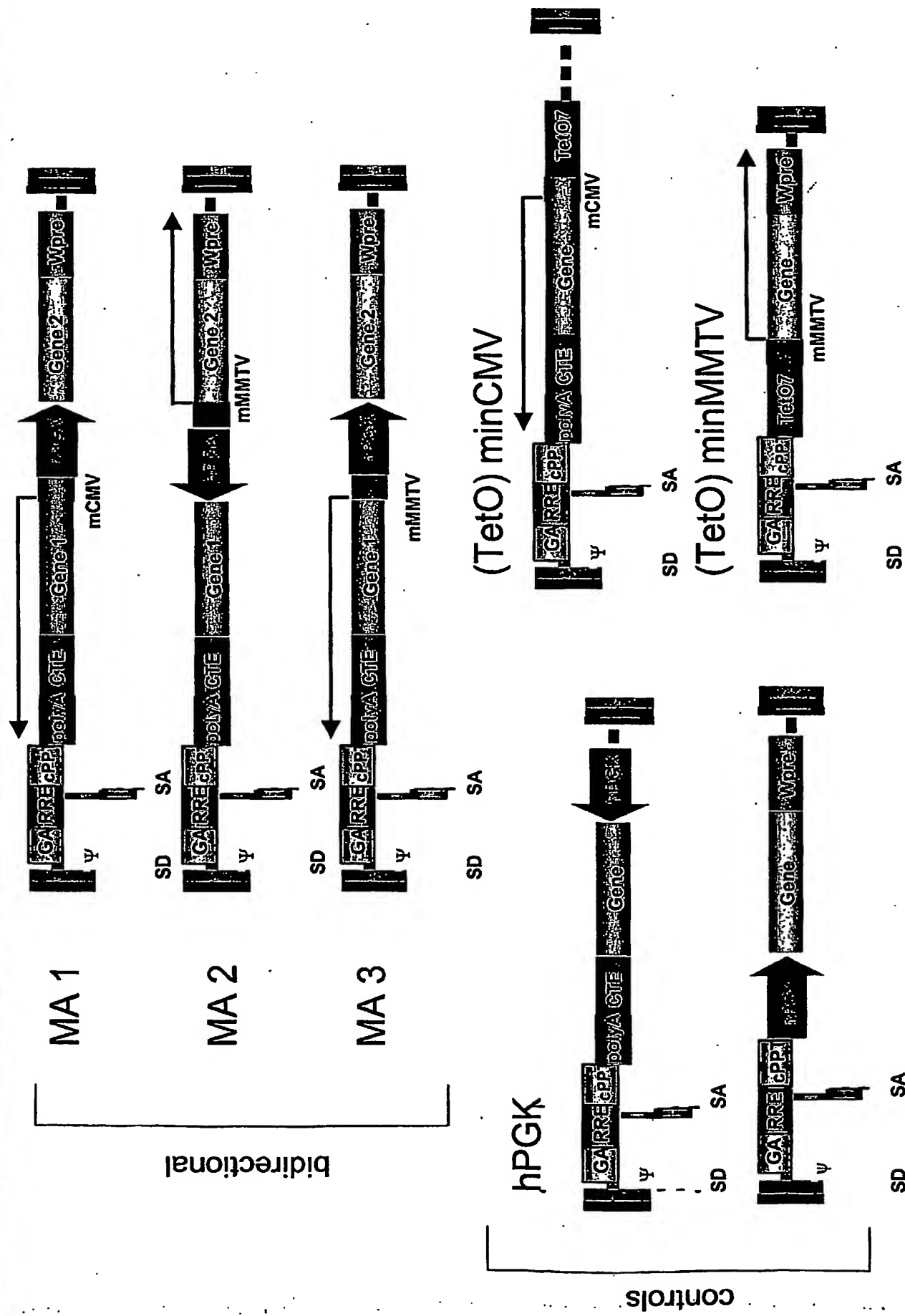


Fig. 7 Bidirectional Vectors: GFP Expression

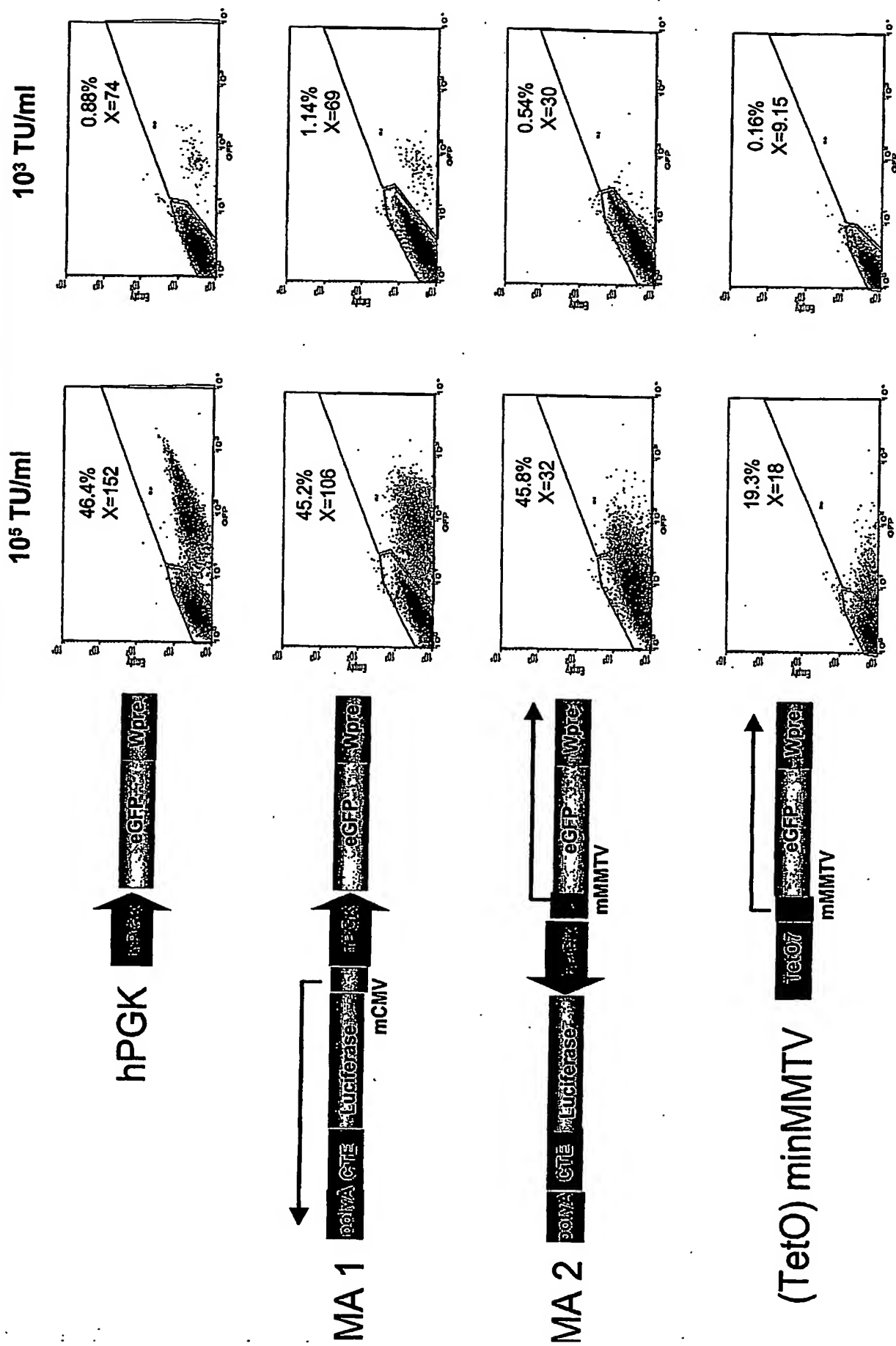


Fig. 8 Comparing Bidirectional and Bicistronic LV

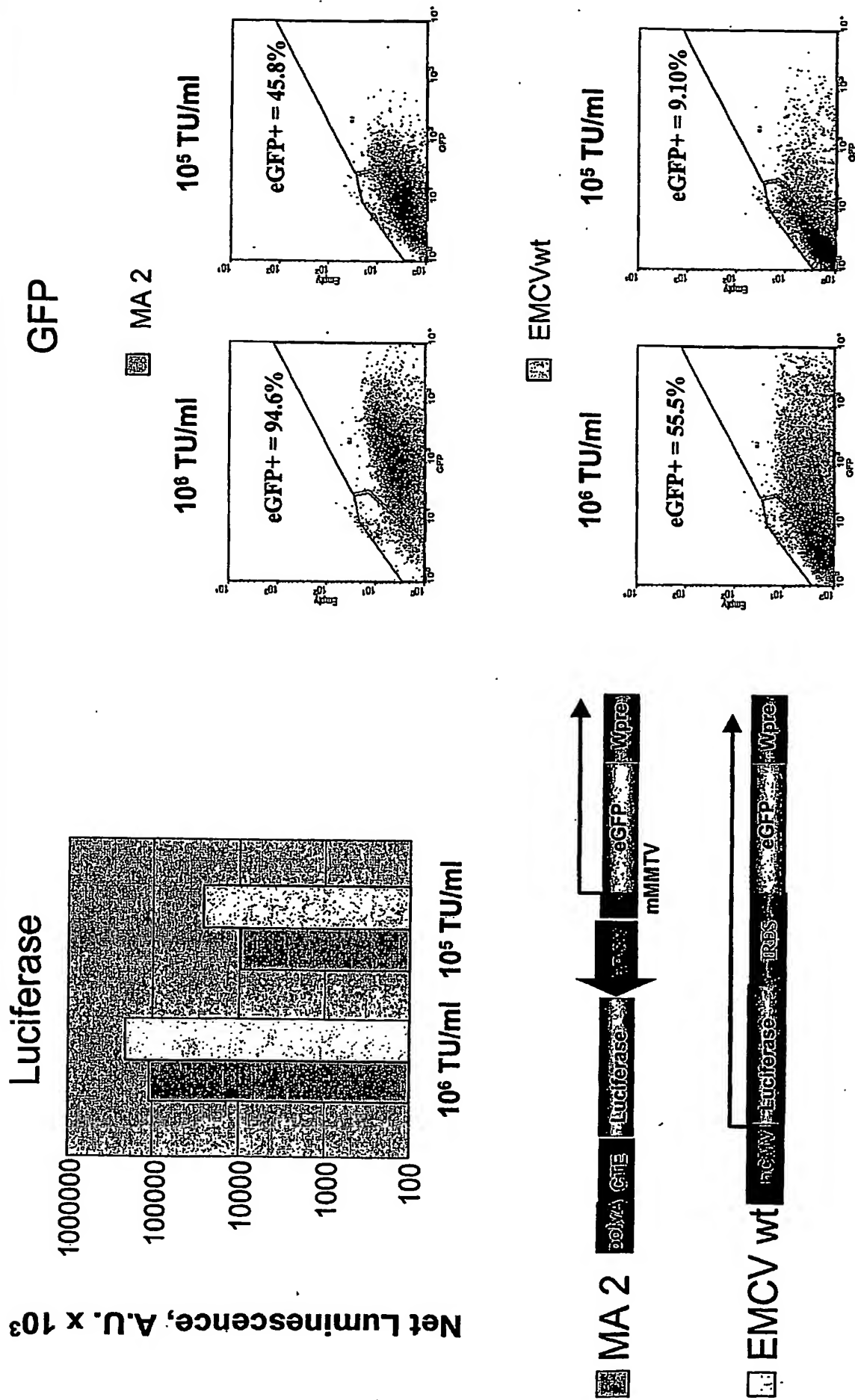
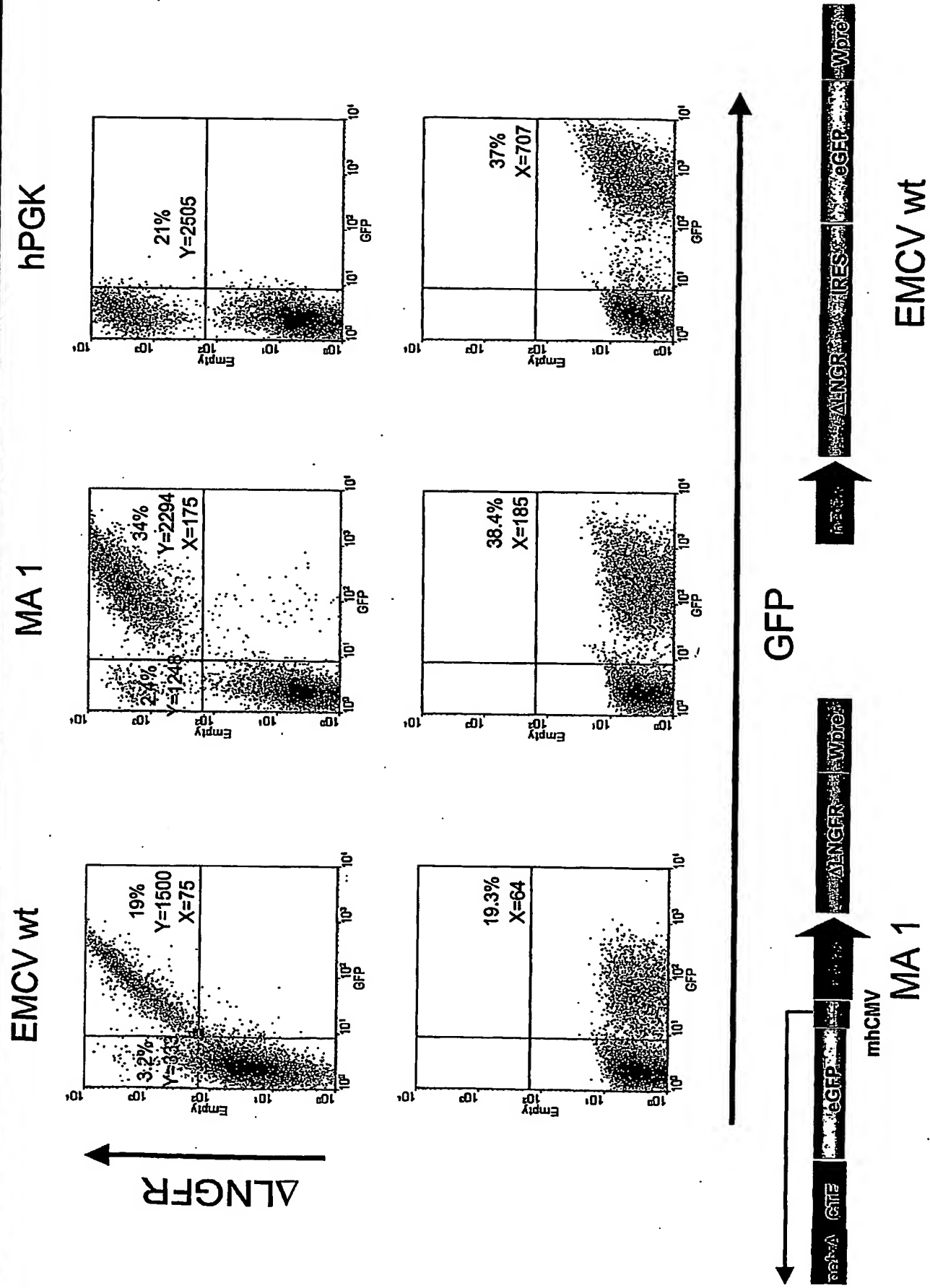


Fig.9 Δ NGFR and GFP expression in CD34 Progenitors



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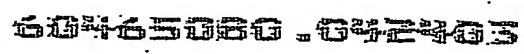
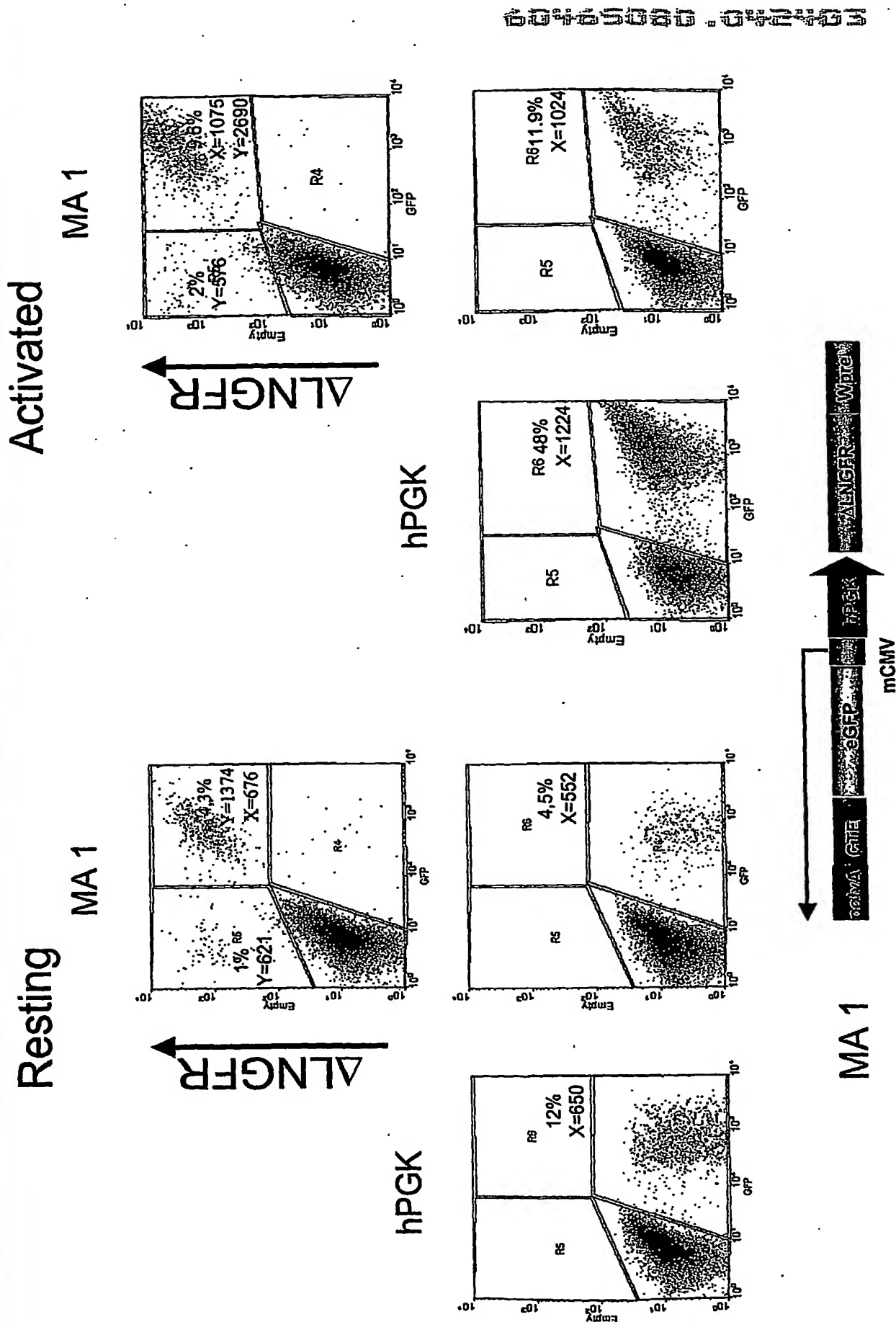


Fig. 11 Δ NGFR and GFP expression in PBL



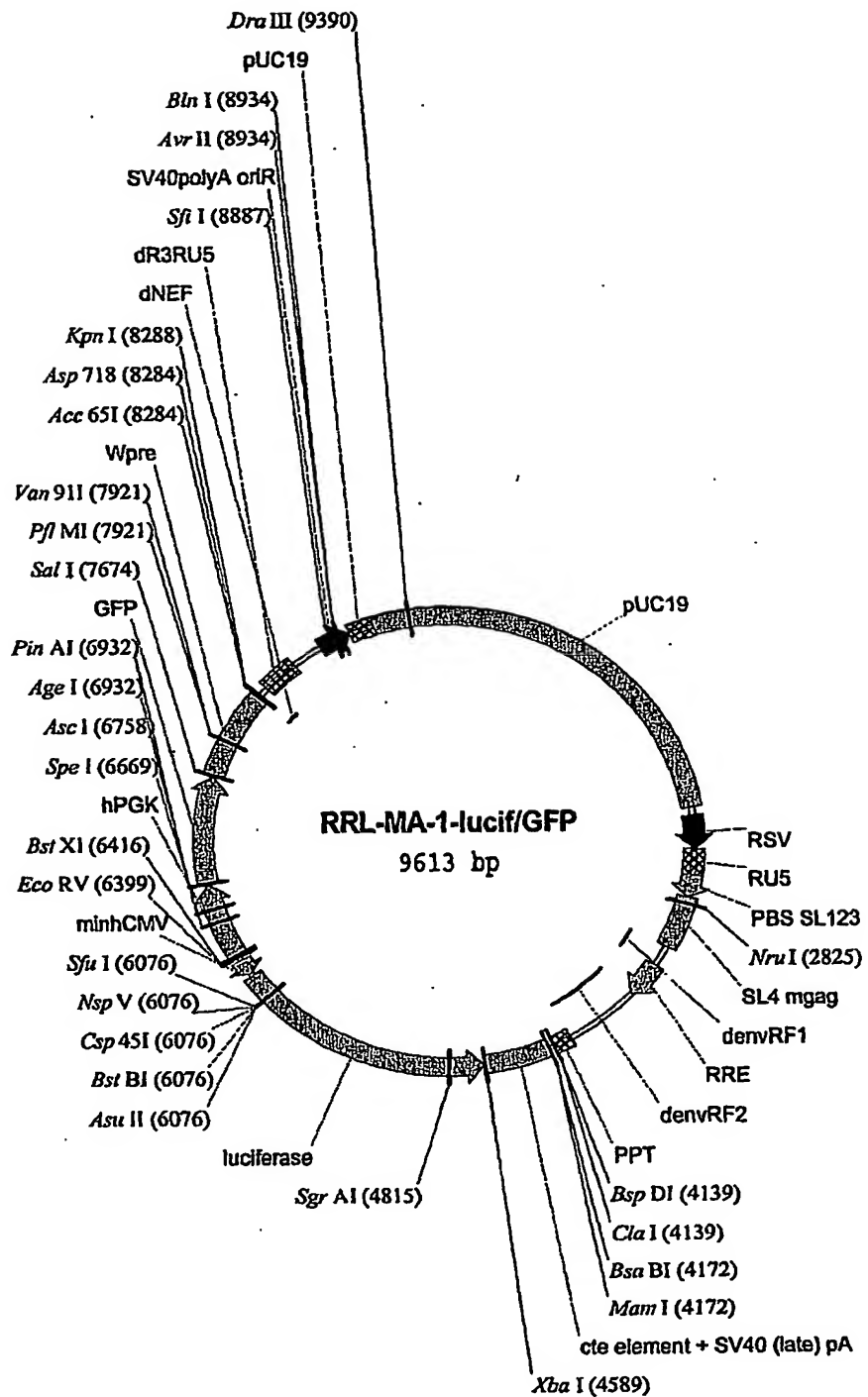


Fig 12a Map of the plasmid containing the lentiviral vector construct RRL-MA1-lucif/GFP

Fig. 12b Sequence of the plasmid containing the lentiviral vector construct RRL-MA1-lucif/GFP

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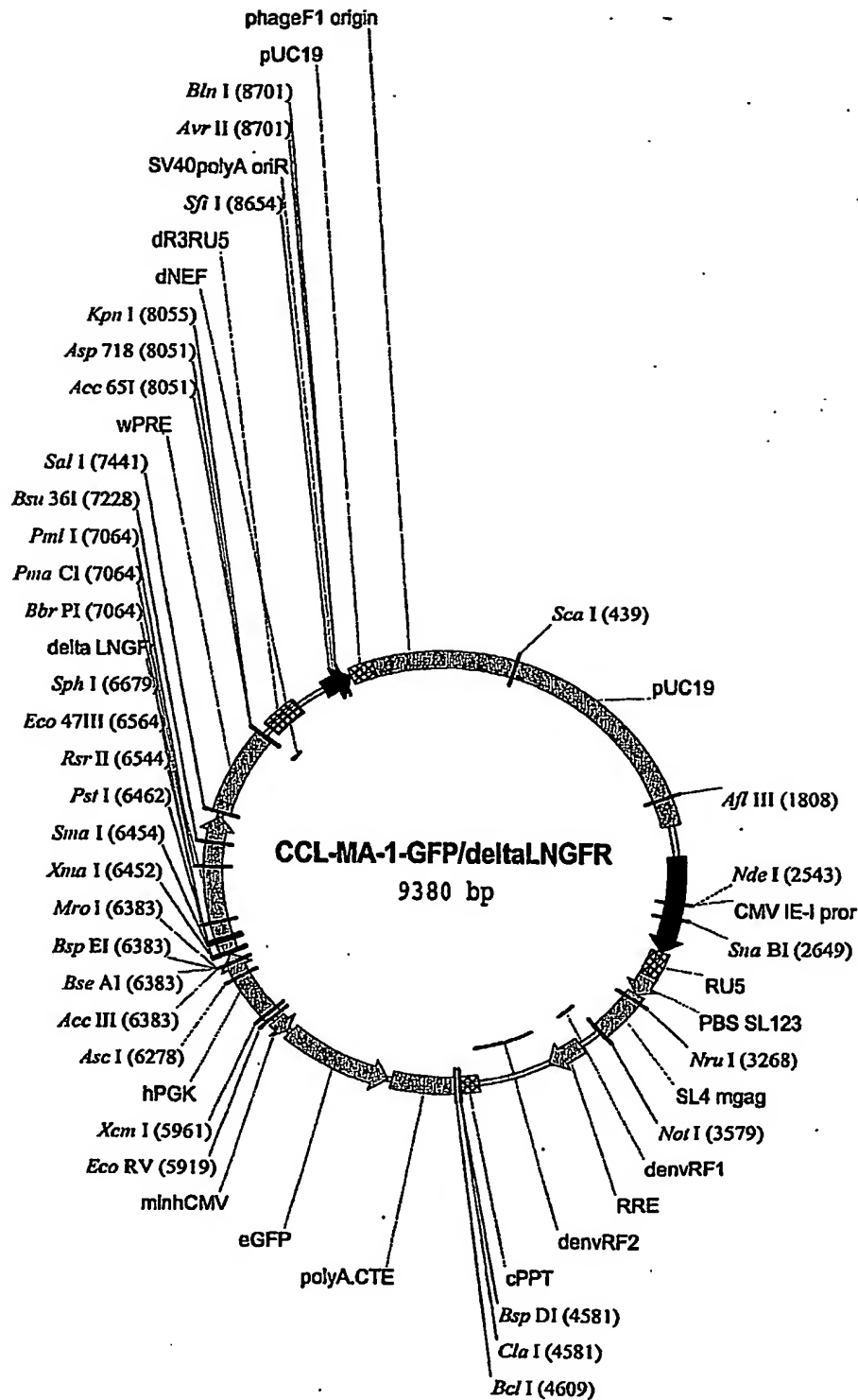


Fig. 13a Map of the plasmid containing the lentiviral vector construct CCL-MA1-GFP/deltaLNGFR

Fig. 13b Sequence of the plasmid containing the lentiviral vector construct CCL-MA1-GFP/deltaLNGFR

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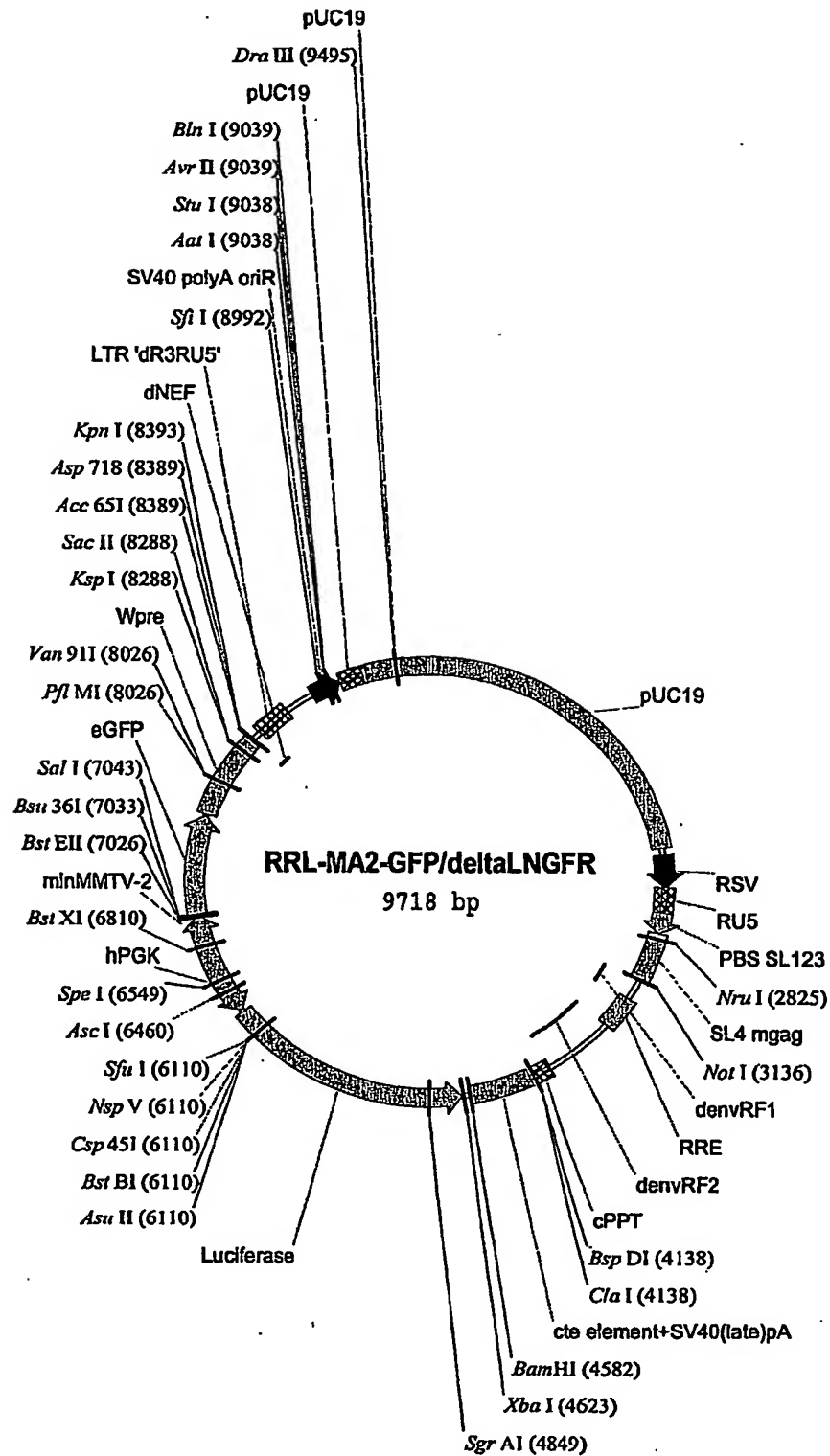


Fig. 14a Map of the plasmid containing the lentiviral vector construct RRL-MA2-lucif/GFP

Fig. 14b Sequence of the plasmid containing the lentiviral vector construct RRL-MA2-lucif/GFP

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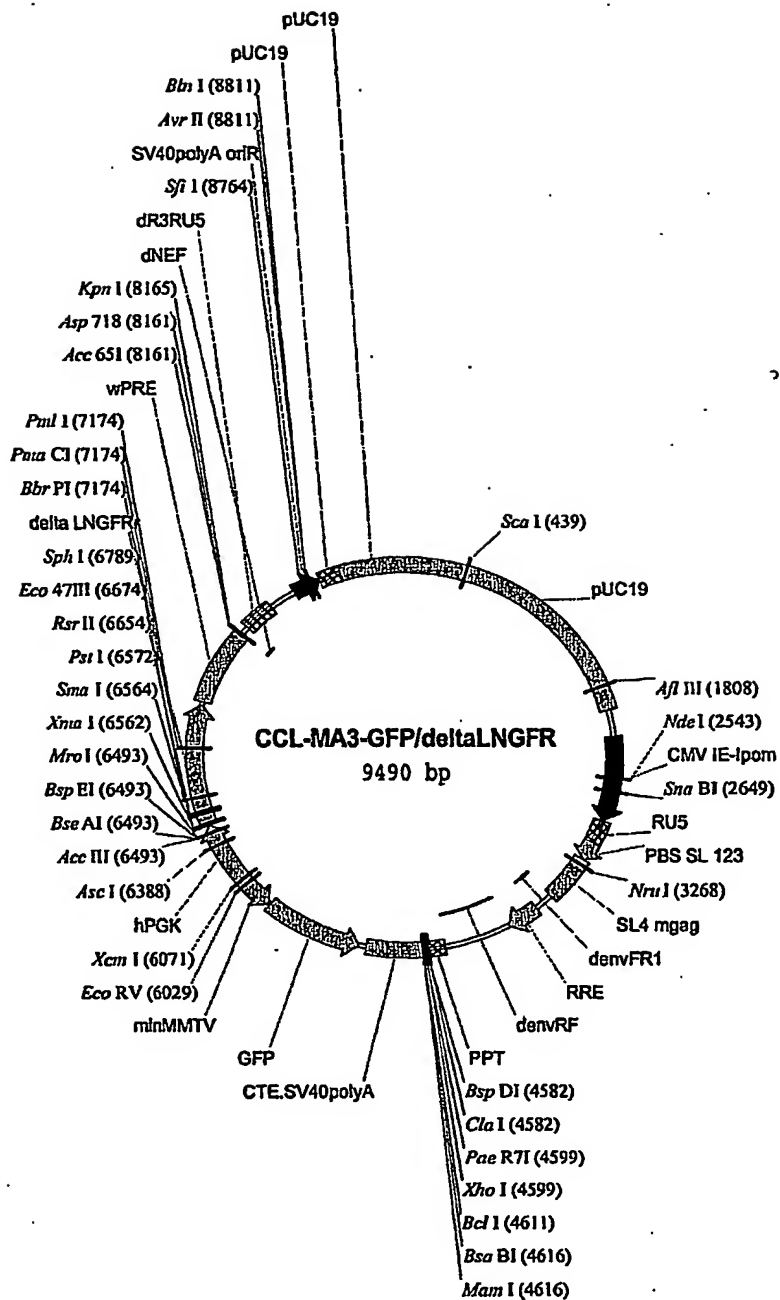


Fig. 15a Map of the plasmid containing the lentiviral vector construct CCL-MA3-GFP/deltaLNGFR

Fig. 15b Sequence of the plasmid containing the lentiviral vector construct CCL-MA3-GFP/deltaLNGFR

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